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Molecular targets of alcohol action: translational research for pharmacotherapy development and screening

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Summary

Alcohol abuse and dependence are multifaceted disorders with neurobiological, psychological, and environmental components. Research on other complex neuropsychiatric diseases suggests that genetically influenced intermediate characteristics affect the risk for heavy alcohol consumption and its consequences. Diverse therapeutic interventions can be developed through identification of reliable biomarkers for this disorder and new pharmacological targets for its treatment. Advances in the fields of genomics and proteomics offer a number of possible targets for the development of new therapeutic approaches. This brain-focused review highlights studies identifying neurobiological systems associated with these targets and possible pharmacotherapies, summarizing evidence from clinically relevant animal and human studies, as well as sketching improvements and challenges facing the fields of proteomics and genomics. Concluding thoughts on using results from these profiling technologies for medication development are also presented.

Keywords

Alcohol; animal models; behavior; biomarkers; complex trait disorders; dependence; drug development; gene expression; phenotype; proteomics

I. Introduction

According to the World Health Organization, there are 76.3 million people with diagnosable alcohol use disorders (AUDs) worldwide. The molecular mechanisms as well as the genetic influences underlying excessive alcohol consumption are not fully understood. Long-term

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alcohol abuse induces persistent alterations in brain function which are manifested through tolerance, physical dependence, craving, and other behavioral changes associated with alcohol. Important risk factors include genetic predisposition, social environment, stress, mental health, age, and the sex of the individual. The proportion of risk for AUDs due to heritability is between 40 and 60%, as deduced from family and twin studies (1). Thus, genetic factors play a critical role in the etiology of AUDs. Nevertheless, some distinctions between these genetic risk factors need to be made.

First, some of the genetic influences are not specific for AUDs, but likely reflect a general vulnerability for polysubstance abuse (2–6). This predisposition may impact the risk for repetitive use of alcohol and other drugs and their associated problems through altered levels of impulsivity, sensation seeking, neuronal disinhibition, and magnified feelings of reward when these substances are self-administered. Genes with polymorphisms potentially linked with these characteristics include GABA (A) receptor alpha 2 (7–9), muscarinic cholinergic receptor 2, alcohol dehydrogenase 4, dopamine receptors D2 (10–13) and D4 (14, 15) and the ACN9 homologue (16).

Second, the propensity to abuse alcohol and other drugs can be affected by vulnerability for other psychiatric conditions such as schizophrenia, bipolar disorder as well as antisocial personality disorder (6, 17–19). A wide range of genes are potentially linked to these psychiatric disorders (20). These disorders are also associated with an increased risk for alcohol and other substance use disorders, possibly through stress mechanisms, attempts to alleviate symptoms of co-occurring disorders or side effects from medications, poor decision-making associated with these disorders, as well as overlapping impairments in neurochemical systems (21–23).

Third, some of the genetic influences on AUDs may be specific for alcohol (2, 6, 24–26). These specific susceptibilities might operate through genes affecting metabolic enzymes, neurochemical pathways or the intensity of a response to alcohol. An example is the low level of response (LR) to alcohol, which is relatively unique to this substance (27) and predicts later heavy drinking and dependence even though it is not associated with repetitive heavy use or problems with any other drug (28–30). Genes with polymorphisms potentially linked with these characteristics include the serotonin transporter, (31–34) GABA A receptor alpha 6 subunit (35) adenylyl cyclase, and the BK potassium channel (36, 37). An exacerbated response to alcohol can also be manifest. Some polymorphisms affect alcohol and aldehyde dehydrogenase enzymes (15, 38) which result in higher levels of acetaldehyde (first metabolite of alcohol), with a subsequent more intense and sometimes aversive response to alcohol, and a corresponding decreased risk for repeated heavy drinking and associated problems. Polymorphisms associated with this lower (or higher in the case of ALDH2) risk for AUDs include mutations of the ADH1B, ADH1C, and ALDH2 genes (15, 38–40).

Even though a relatively large amount of information is known about genetic factors underlying a risk for AUDs, most of the medications currently used have provided modest results. In the past decade, advances in our understanding of complex trait diseases have increased dramatically as a result of genome-wide studies. The challenge for post-genomic

biologists is to systematically utilize these vast datasets to predict the cellular processes involved. These predictions rely on “omic” studies of gene transcripts and proteins, as well as their regulation. The hypothesis is that these predictions will provide new pharmacological targets leading to the development of new medications for alcohol dependence.

The following sections focus on the molecular targets of alcohol’s action in the brain with a spotlight on translational aspects, beginning with current treatments, the importance of improved therapeutic intervention, and progressing to a more detailed survey of preclinical evidence from gene and protein expression studies and their associated molecular mechanisms. Finally, some considerations for future directions will be presented, including the impact of cutting-edge technologies on the screening of new targets and associated treatments.

II. Alcohol abuse and dependence, significance, and treatment needs

Alcohol abuse and dependence are conditions influenced by multiple factors, including genetic, neurobiological, psychological and environmental components (6, 19, 41–43). Chronic alcohol abuse results in the development and expression of tolerance to alcohol’s effects, symptoms of withdrawal upon the removal of alcohol, and compulsive behavior focused on obtaining more alcohol. Clinically, the condition is diagnosed when these behavioral changes are manifested and lead an individual to “forsake” occupational, familial, social, and other important responsibilities, as outlined in the Diagnostic and Statistical Manual, 4th edition (44).

The treatment of alcohol use disorders (AUD) represents a main goal in public health, but the currently available, Food and Drug Administration-(FDA) approved medications are limited and have mixed efficacy in the heterogeneous clinical population (42, 45–48). Presently, the FDA has approved only 4 medications for the treatment of AUDs: These are disulfiram, an aldehyde dehydrogenase inhibitor (*Antabuse*™), acamprosate, a functional glutamatergic antagonist (*Campral*™), and two of these are based on naltrexone, a pan-opioid antagonist (*ReVia*™, oral form, and *Vivitrol*™ for depot administration) (49–51). Thus, there is a critical need to identify new pharmacological targets in order to improve the spectrum of medications for treating AUDs. Indeed, individuals with AUDs represent a heterogeneous population and pharmacogenetic studies support this and will provide personalized treatments in the near future.

Continued research on alcohol abuse and dependence has identified several neurobiological systems that have revealed new neuropharmacological targets that continue to be investigated (48). These neurobiological systems include corticotropin-releasing factor (CRF), endocannabinoid, neuropeptide Y, substance P, nociceptin, alpha-adrenergic, glutamatergic, nicotinic cholinergic, neuroinflammatory, acetaldehyde-related enzymes, and other systems (43, 48). It has also been suggested that feeding-related pathways contribute to alcohol-seeking behavior, and peptides such as leptin (52), insulin (53), thyroid hormones (54), and ghrelin (55, 56) are being studied to pharmacologically mimic or counteract alcohol’s effects.

These preclinical efforts have resulted in a number of clinical trials that are providing important feedback from the clinical arena to guide continued preclinical research. Some of these clinical trials are examining aripiprazole (57), topiramate (58), baclofen (59), ondansetron (60), and varenicline (61). While feedback from the clinical field is providing important information to direct preclinical research efforts, the development of new methods of preclinical inquiry promise to accelerate the identification and development of new pharmacological treatments targeting AUDs.

Advances in the field of genomics offer new diagnostic and screening potential even for complex genetic diseases like alcoholism. The importance of understanding gene expression changes during and/or following alcohol abuse and dependence can be appreciated by the impact of expression profiling in other diseases, most notably cancer, where studies have led to improved pharmacotherapies (62–64) and to a molecular classification of diseases that promises to be more accurate and informative than traditional diagnostic tests (65–68). Thus, the application of gene expression profiling to psychiatric disorders (69–72) should provide more accurate means to diagnose these conditions as well.

Importance for identifying biomarkers of alcohol abuse

Although the term has often been improperly used to refer to gene or protein expression change, a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (73).

The availability of rapid and accurate tests for the early detection of disease states plays a critical role in treatment outcome. Thus, early diagnosis of complex diseases such as alcoholism may benefit from large-scale analyses to identify disease-specific molecular markers that provide a fingerprint of the condition. Unfortunately, alcoholism is a major public health problem that is often overlooked by primary care physicians. In part, this is due to the fact that there are no biomarkers that screen for alcohol dependence and use and maintain high reliability across the progression of the disease. Existing blood tests for alcohol dependence, such as carbohydrate deficient transferrin (74), have limited capabilities and are not widely used. Development of a reliable molecular blood test for alcohol dependence and heavy drinking would be a milestone in the diagnosis and ultimate treatment of this disease (see “Translational strategies and need for continued biomarkers development” section below).

III. Preclinical strategies for identification of novel targets of alcohol action

Alcohol abuse produces persistent changes in brain function that result in tolerance to its effects, physical dependence after chronic use, craving during and/or prior to relapse and other behavioral changes. These changes in brain function likely originate from alterations in gene and protein expression underlying the cellular adaptations associated with chronic alcohol abuse (65, 75). The importance of understanding changes in multiple response variables associated with alcoholism can be appreciated by the impact of findings from gene and/or protein expression profiling of other diseases, most notably cancer, where studies have led to improved pharmacotherapies (62–64) and to a molecular classification of

disease-states which promise to be more accurate and informative than traditional diagnostic tests (76–79).

Gene and protein expression profiling techniques are only beginning to be applied to psychiatric disorders (80–82) and it is not clear, at this point, if these approaches can provide the more accurate classifications as seen with cancer and other diseases. Initial studies indicate that changes in brain gene expression in schizophrenia (69) and alcoholism (83) are much smaller than those seen in cancer (84, 85) and it is possible that individual differences due to other factors will overshadow the differences found in these disorders. In view of these concerns, a molecular classification is most likely to succeed if expression data for a large number of genes are combined with appropriate statistical tests and bioinformatics. The genomic and proteomic fields have matured rapidly over the past several years, and important advances in bioinformatics will undoubtedly improve our ability to interpret large data sets with common underlying themes.

Even though it is possible that a limited number of genes might directly account for the development and expression of AUDs, general studies from other complex neuropsychiatric diseases suggest that it is more likely that the relevant genes influence a range of endophenotypes (86), i.e., genetically influenced intermediate characteristics that affect the risk for developing heavy drinking and alcohol dependence (3). Each endophenotype reflects the actions of multiple genes and is influenced by gene-by-environment interactions (30, 87). Some endophenotypes important for understanding alcohol abuse and dependence include a genetic predisposition for alcohol abuse (88), an enhanced risk for polysubstance abuse (e.g., presence of DRD2 or DRD4 alleles) (89), a vulnerability for impaired neurobehavioral or neurobiological effects of stress (90), and a low level of response (LR) to alcohol (91), which increase the risk for repeated heavy drinking (6, 92). A substantial part of the research for specific human genes that affect the risk for developing alcoholism has focused on genetic influences related to intermediate endophenotypes. Potentially, this may be more successful than looking for genes influencing broader substance-dependence-associated phenotypes (6, 88, 93–99).

For most characteristics, sufficient information on how a genetic phenotype, or a specific polymorphism, impacts an individual’s vulnerability to develop an AUD can only be achieved when evaluated within the environment in which the gene(s) operates. About 40% of the variance of risk for alcohol abuse and its associated problems can be explained through gene-by-environment interactions associated with family, alcohol expectancies, socioeconomic status, education, peers, stressors, etc. (6, 100). Thus, a better understanding of how these interactions operate will help with the identification of individuals at risk for alcoholism and provide important approaches to prevention and/or treatment. A complete review of this complicated area is beyond the scope of this review; nevertheless, additional work is needed to evaluate the role of environmental factors in the development of alcohol- and drug-dependence.

Challenges in the investigation of complex trait disorders

The extraordinary success of the molecular revolution in advancing modern biology has generated the important problem of bioinformatics. Essentially, how can the large amount of

molecular data that is being generated be synthesized to gain insight into higher-order processes at the levels of neurobiological pathways, organ systems and whole organisms (101). Understanding the genetic basis of variation for quantitative traits is a major challenge in current biology (102). Complex or quantitative traits are those influenced by multiple loci (genes), each of which is known as a quantitative trait locus (QTL). To date, many studies mapping QTLs associated with human diseases and complex traits have uncovered new loci and provided unexpected insights into the biology of diseases (102). Regarding alcohol-associated behaviors, rodents have been used extensively to study alcohol-related phenotypes and the behavioral genetics of alcohol's action (88, 91, 103). These studies show that differences in alcohol-associated sensitivity is under some genetic control and, in general, there is a heritability range of 0.2–0.5 (104). Crosses between inbred strains are useful and valuable tools for determining which chromosomal regions control these genetic differences in alcohol-associated sensitivity. These studies usually require a large number of different recombinant inbred strains or individual F2 generation animals. Therefore, genetic tools such as QTL maps are readily available for mice and to a lesser extent for rats (88).

Differences in the magnitude of alcohol-associated sensitivity across these strains (or individuals) and their associated differences in DNA sequences (single nucleotide polymorphisms, SNPs) can be determined. A correlational analysis of this information establishes links/associations between chromosomal regions (loci) and the behavioral trait under investigation (6). One goal of QTL mapping is to determine the gene(s) responsible for the QTL, that is, the quantitative trait gene. Published QTL maps for different alcohol-related behaviors include acute functional tolerance, loss of righting reflex, taste aversion, withdrawal severity, voluntary consumption and conditioned place preference (103, 105–107). Nevertheless, for the behavioral effects of alcohol, this time- and resource-intensive process has been completed to the greatest extent for alcohol withdrawal severity (6, 108). As a general assumption, at least some of the QTLs reflect differences at the gene expression level rather than differences in coding region (protein) sequence. Gene expression profiles derived from microarray analysis can be compared across recombinant inbred strains or other genetic tools, and genes from QTLs with differential expression are likely to provide promising candidate genes in this endeavor (6, 109). Indeed, this was recently obtained for the alcohol-induced loss of righting reflex, as well as for alcohol preference and acute functional tolerance (110, 111). Several mouse candidate genes are also located in human alcohol sensitivity-associated QTLs (110). The behavioral, gene expression and gene-sequence differences among recombinant inbred strains are being cumulatively added to online databases as strains are being tested for different phenotypes. These resources provide powerful online platforms for in silico analyses of relationships among these three variables (112, 113).

Despite the substantial progress resulting from these tools, it should be emphasized that these loci account for only a small fraction of the total genetic variation associated with alcohol-induced effects and do not map to individual genes (102). The main problem is not the intellectual foundation of QTL mapping methods but the technological limitations. The present genomic revolution and its development of economical, massively parallel technology for DNA and RNA sequencing as well as platforms for rapidly genotyping

hundreds of thousands of polymorphic markers, has begun (102). With the advances of technologies and the increased affordability, larger sample sizes, more developmental time points, tissues, and environmental conditions can be analyzed by systems genetics. Moreover, these advances allow for an unbiased sampling and evaluation of the transcriptome. This is important because not all functional molecular polymorphisms exert their effects on organismic traits through measurable alterations in gene expression. Combining information on qualitative and quantitative variation in the expression of proteins and metabolites, as well as epigenetic modifications, will yield a more complete picture of the effects of genetic perturbations on the whole organism (102).

A rationale for the “omic” approaches

Traditionally, a single or at most a few transcripts have been studied at a time. The development of the new technologies mentioned above offers distinct advantages over “traditional” molecular strategies, when investigating complex issues such as the search for genes and proteins that are affected by alcohol or that mediate alcohol’s effects. Despite the difficulties inherent in the available genomic and proteomic technologies, they have changed the way genes and proteins are studied, with many investigators currently using expression profiling to define global transcriptomes from different tissues. For example, microarrays have been used to identify alcohol-induced changes in gene expression in both cultured cells and animal models of alcoholism (114–125). Such “omic” approaches have proven to be valuable tools in the search for the genetic foundation for complex diseases such as cancer, neurodegeneration and drug abuse, because they allow researchers to examine large numbers of potential target molecules (e.g., RNA transcripts and/or proteins) simultaneously and in an unbiased fashion without having to make a priori assumptions about which molecules might be involved. Nevertheless, despite the fact that genomic and proteomic techniques have matured rapidly over the past few years, the analyses of the plethora of data generated from such studies have proven to be challenging. The integration and interpretation of huge amounts of data obtained from these studies are complicated by many factors, such as, the microarray platform used, the specific features (genes/clones) represented on the array, the statistical analyses and the gene selection strategies used for identifying significant changes in the genes. The use of a rigorous and well-informed bioinformatic approach is crucial for interpretation of the results. Confirmation of changes in mRNA or protein expression with alternative techniques is also critical. Important advances in bioinformatics will undoubtedly improve researchers’ ability to interpret the large amounts of accumulating data and identify common underlying themes. This then may lead to the identification of alcohol-sensitive genes and proteins as well as the biological systems in which they exert their effects and increase our understanding of mechanisms of action for drugs of abuse, including alcohol. In turn, this information can guide the development and/or screening of pharmacotherapies targeting AUDs, which has already been demonstrated in cancer research with improved pharmacotherapies (62–64). Just as behavioral, gene expression and gene-sequence differences among recombinant inbred strains are being cumulatively added to online databases for in silico analyses involving QTLs as discussed earlier, this information is also being compiled and being made available as it pertains to protein and/or gene expression changes.

Molecular targets of alcohol action

Chronic alcohol exposure leads to a wide range of modifications in brain function. Alcohol damages neurons in different brain regions, leading to cognitive impairment and other abnormalities of brain function in alcoholics (126–128). At the same time, the brain adapts itself to the constant presence of alcohol, counteracting alcohol's negative effects, and this neuronal and behavioral adaptation can lead to severe withdrawal symptoms if alcohol levels suddenly drop. Both alcohol's damaging effects on the brain and the body's adaptive responses are most likely mediated, at least in part, by altered gene and protein expression (75). Nevertheless, genes and their associated proteins do not necessarily follow a parallel trend in expression levels. In addition, the expression of both of these molecules can be regulated by microRNAs. A plethora of studies have investigated these alterations with various techniques, and the following sections provide a survey of findings from many of these studies (see Tables 1 and 2).

Gene expression studies of clinical relevance

Human post-mortem brain studies: Many studies have listed important quality control guidelines that should be considered when performing expression profiling experiments on the human post-mortem brain (129–131). The post-mortem interval, agonal state and pH are all important variables that affect transcript quality, but high quality RNA can still be obtained and expression profiling of post-mortem human brain has been successfully applied to a number of neurological disease conditions, including Alzheimer's disease (132, 133), multiple sclerosis (134, 135), and Rett syndrome (136). Furthermore, psychiatric disorders such as major depression (137), bipolar disorder (138), schizophrenia (69, 139, 140), and autism (141) have been studied successfully as well. The results from these studies indicate that numerous genes have altered expression levels and that identification of such changes using the traditional "single target" approaches would be inefficient and have limited benefit.

Comparisons across human post-mortem brain gene expression studies performed from different laboratories are complicated by a number of factors. Differences in tissue harvesting, array platforms, sample processing and labeling, and data analysis procedures make post hoc comparisons of microarray data a great challenge (142). Here, we should not only consider differences in the brain regions studied, but also differences in case selection criteria such as age, gender, smoking history, alcohol drinking history, etc. Accurate clinical information is critical in the experimental design and for evaluating individual differences across cases. Indeed, case grouping is often determined by using variables such as the amount of alcohol consumed per unit of time and/or diagnostic criteria standardized for different cultures, geographic regions, ages, etc.

Differentially expressed genes in response to long-term alcohol consumption have been studied by using gene expression profiling in several cortical brain regions (83, 143–148) (Table 1). The most often studied region is the prefrontal cortex (PFC), an area crucial for judgement, decision-making, and other cognitive functions (149–151) that are often impaired in alcoholics because of this region's increased susceptibility to damage by alcohol. This region is also associated with neurocircuitry mediating reward, which

influences the development and expression of alcohol tolerance and dependence (152). Early gene expression profiling studies on the PFC consistently identified functional groups of differentially expressed genes involved in myelination, protein trafficking, ubiquitination and mitochondrial function (143, 144, 147, 148). Nevertheless, the individual genes identified often differed in their direction and magnitude of change across studies. These variations were probably due in part to differences in the array platform and/or experimental design, since some studies either used RNA pooled from several individuals (143, 144) or relatively small sample sizes (147).

More recently, rigorous statistics were applied to identify differentially expressed genes in the PFC from 27 subjects (14 well characterized alcoholic cases and 13 matched controls) (83). Similar to the earlier studies, genes with altered expression levels included those involved in myelination, ubiquitination, apoptosis, cell adhesion, neurogenesis and neural disease. Presenilin 1 and transferrin genes were also significantly altered, and, since these genes are involved in neurodegenerative diseases such as Alzheimer's, a link between alcoholism and other neurodegenerative conditions can be hypothesized. Results from other independently published expression studies with post-mortem brain tissue (143–145, 148, 153) were used to verify a total 232 candidate alcohol-responsive genes identified in this study, by comparing the magnitude and direction of change with the previous studies. Out of the 232 identified genes, 27 were also differentially expressed in these previous studies. Of these 27 genes, 21 were regulated to a similar extent and in the same direction as the Liu et al study (83). Interestingly, myelination-related genes transferrin (143), UDP glycosyltransferase 8 (143), peripheral myelin protein 22 (143, 148), and proteolipid protein 1 (143) were downregulated in multiple studies.

Other differentially expressed genes identified by Liu and colleagues (83) and confirmed from previous studies included lysosomal-associated membrane protein 2 (145, 148), proteasome subunit, β type 2 (148), CANX (145), GABBR1 (148), solute carrier family 12, member 2 (144), and transketolase (153). Another important discovery of this study (83) was the identification of a group of 20 cell adhesion genes, 18 of which were down-regulated. Since these genes play a role in CNS development, synaptic formation and immune responses (154–158), their down-regulation may lead to impaired neuronal function in the alcoholic human brain. Finally, Liu and colleagues (83) used a principal component analysis to investigate individual variability in gene expression patterns to discriminate alcoholics from non-alcoholic controls. Using functionally related sets of genes, controls and alcohol-dependent cases could be predicted with the exception of three misclassified cases, which turned out to be a subject with polysubstance abuse, an alcoholic subject that had been abstinent for 2 years, and a subject that had been undergoing treatment for depression. These latter findings highlight the importance of detailed clinical information for an accurate assessment of individual variation in gene expression patterns.

Analyses of post-mortem brain of long-term alcohol abusers have reported neuronal loss in grey matter and loss of white matter volume (126, 127, 159–161). These devastating effects are not as severe in motor, temporal or cingulate cortices, suggesting that alcohol-induced brain damage is potentially selective to brain regions. To determine the regional specificity of alcohol-related reprogramming of gene expression, it is important to compare the pattern

of differentially expressed genes from different brain areas. There are two studies (144, 147) where the expression profiles from the PFC and motor cortex (MC) were compared, and another study in which gene expression changes were examined in the temporal cortex (145). As outlined for the PFC above (144, 147), differentially expressed genes generally fell into the same functional groups, in comparisons between the PFC and MC.

The first study by Liu et al. (147) utilized individual cases rather than pooled samples and reported only 5% overlap in differentially expressed genes between the PFC and MC. An additional study by Flatscher-Bader et al. (148), compared distinct regional effects of alcohol in the PFC and the nucleus accumbens (NAc), two key brain regions of the mesocorticolimbic reward neurocircuit, which plays an important role in mediating the rewarding effects of addictive drugs (162). Approximately 6% of genes with a marked alcohol response were common between the PFC and NAc, a degree of overlap similar as that seen between the PFC and MC discussed above. These findings suggest that there is remarkable regional heterogeneity in the expression patterns of individual genes following chronic alcohol abuse. In the Flatscher-Bader and colleagues study (148), the differentially expressed genes in the PFC were associated with DNA-binding proteins (transcription factors and repair proteins), and genes encoding mitochondrial proteins or those associated with neuroprotection/apoptosis. On the other hand, genes involved in synaptic vesicle formation and regulation of cytoarchitecture were significantly down-regulated in the NAc. These authors suggested that the gene expression changes observed in the PFC could result in disrupted mitochondrial function and energy production leading to oxidative stress, whereas the changes in gene expression in the NAc might result in a persistent deficit in synaptic transmission and changes in neuroplasticity resulting in neuroadaptations at the level of synaptic structure and function (148).

The impact of concomitant liver cirrhosis on brain gene expression has been tested by profiling expression patterns in the frontal cortex (FC) of cirrhotic (CA) versus non-cirrhotic alcoholics (NCA) (163). Cirrhosis, a common concomitant condition resulting from long-term alcohol abuse, is the widespread disruption of normal liver structure by fibrosis and the formation of regenerative nodules. Importantly, the magnitude of change in gene expression levels between CA and NCA was much greater than that observed between NCA and controls. Among the down-regulated genes, those involved in cell adhesion, mitochondrial function, and synaptic transmission were over-represented, whereas among the up-regulated, transcripts involved in apoptosis and mitosis were over-represented. Neurotoxins such as ammonia can pass through the blood-brain barrier and affect brain function, and their main targets in cirrhotic patients are the astrocytes (164–167). Astrocytes play an important role in the proper function of the CNS by providing basic structural support, producing trophic factors for neurons, maintaining the concentrations of ions and neurotransmitters in the extracellular space, and removing neurotoxins and cellular debris from this space as well (168). To study the possible effects of cirrhosis on glial cells at the transcriptional level, the expression levels of astrocyte-specific transcripts in the CA were compared with those in NCA (163). Genes associated with astrocytes such as reticulon 4, ATP-binding cassette, sub-family A, member 1, apolipoprotein E and microsomal glutathione S-transferase 1 were all up-regulated in CA, whereas other transcripts such as aquaporin 4, transmembrane 4 superfamily member 2 and phosphoprotein enriched in astrocytes were down-regulated in

CA. This gene expression profiling study shows that concomitant liver cirrhosis may specifically alter gene expression in astrocytes during long-term alcohol abuse. This disruption may result in neurodegeneration as discussed above. Thus, alcohol-induced alterations in peripheral tissue can influence CNS function and/or influence alcohol's effects on CNS function.

In summary, gene expression profiling in the human alcoholic post-mortem brain shows a regional-specific transcriptional reprogramming at the level of individual genes. These changes at the transcriptional level may reflect both pre-existing differences in gene expression and those altered as a consequence of alcohol consumption. Understanding the exact role that these widespread changes play in cellular regulation during alcohol dependence continues to be a challenge for addiction biologists (6).

Animal phenotypes used for gene mapping and expression: Rodents are the prime organisms of choice for modeling human diseases. The tendency of some rodents to prefer and others to avoid alcohol solutions has provided the cornerstone for behavioral neuroscience research into alcohol use disorders since their discovery more than 60 years ago (91, 103). Behavioral genetics gained a prominent role in preclinical research on alcohol's effects after early evidence that those drinking differences were most genetically based. Since the 1950's and 1960's, selected lines of rats and mice as well as inbred strains of mice that differ significantly in alcohol-related phenotypes have been used to identify genetic and environmental factors underlying individual differences in responses to alcohol (87, 169–174). As genetic, genomic, and bioinformatic tools have proliferated and became much more sophisticated, substantial progress toward identifying some of the genes, and/or systems, responsible for differential avidity for alcohol solutions has been achieved (114–116, 170, 172). This genetic information has led to a resurgence in research on pharmacotherapy development and screening of compounds targeting alcohol abuse and dependence (91).

The observation that people with very similar environmental backgrounds often differ considerably in alcohol consumption indicates that heredity contributes to the development of alcohol abuse and dependence (175–177). Similarly, different lines of outbred rats exhibit a wide-range of alcohol-consumption levels (178), suggesting, as in humans, the propensity to ingest alcohol is genetically influenced. In the late 1940's, Williams and associates (179) and later Mardones and colleagues (180) proposed that high alcohol consumption by rodents was under genetic control. Subsequently, reports that certain C57 substrains (most notably the C57BL6/J) of inbred mice display high alcohol consumption, while DBA2/J inbred mice display low alcohol consumption, supported a genetic influence on alcohol intake (181–183). As seen in clinical research on alcoholism, secondary traits may influence these differences in alcohol-drinking behavior. For example, there is evidence that taste factors, and genes associated with these taste factors, exert substantial control over the alcohol-drinking phenotypes of C57BL6/J and DBA2/J mice (184–187).

However, it has been argued that inbred strains have their limitations when examining "correlated traits and responses" (91, 188–191). In other words, their high alcohol-consuming behavior alone does not necessarily indicate addictive behavior, but often

reflects controlled, albeit excessive, alcohol consumption. This is because inbreeding results in fortuitous (chance) fixation of genes associated with the phenotype being examined. These authors suggested that selective bi-directional breeding, for alcohol preference versus nonpreference, is a powerful research tool for examining factors affecting excessive alcohol intake.

The common inbred mouse strains have been divided into seven general categories (192) based on pedigree records and recent extensive SNP comparisons (193). A library of genetic-marker polymorphisms is available for many strains, including A/J, C57BL/6J, DBA/2J, BALB/cByJ, C3H/HeJ, NON/Lt, LP/J, AKR/J, and FVB/NJ (101). Among these general divisions, the highest drinking mice belong to the strains from the C57/C58 lineage, while those related to the DBA lineage are alcohol-avoiding (194). Recently, Blednov and colleagues discovered that an F1 hybrid between the C57BL/6J and FVB/NJ inbred strains is able to drink even more ethanol than C57BL/6J mice, in standard 2-bottle tests (195). Interestingly, other strains in the FVB (Swiss) lineage usually show wide variability in their alcohol consumption levels, with an overall average drinking phenotype (91, 196).

Through bi-directional selective breeding, five high and low alcohol-consuming lines of rats have been developed. Bi-directional selection, from a heterogeneous foundational stock, is accomplished through systematic mating of animals from the same extreme of the normal distribution over successive generations to obtain divergent lines that display these extremes. This breeding protocol results in selectively bred lines that display high or low alcohol-drinking phenotypes based solely on selection history, i.e., the animals display this behavior without environmental manipulations to induce alcohol consumption and preference over water. The ALKO alcohol-accepting AA and ALKO alcohol-nonaccepting ANA rats were developed from a Wistar foundational stock in Helsinki, Finland (188). The alcohol-preferring P and alcohol non-preferring NP lines of rats were developed by mass selection from a Wistar foundational stock at Walter Reed Army Hospital and transferred to Indiana University School of Medicine in Indianapolis, Indiana, USA (197).The high alcohol-drinking HAD and low alcohol-drinking LAD lines of rats were developed using a within-family selection and rotational breeding design (which decreases the level of inbreeding) from N/NIH heterogeneous stock rats at Indiana University School of Medicine in Indianapolis, Indiana, USA (198). The Sardinian alcohol-preferring sP and Sardinian alcohol-nonpreferring sNP rats were developed from a Wistar foundational stock at the University of Cagliari, Italy (199). The alcohol-preferring (UChB) and alcohol-nonpreferring (UChA) lines of rats were developed from a Wistar foundation stock at the University of Chile, Santiago, Chile (180). All of these alcohol-preferring rat lines display high alcohol consumption, achieve pharmacologically relevant blood alcohol concentrations (BACs) during free-choice access to alcohol, and exhibit many other behavioral and neurobiological phenotypes found in family history positive (FHP) for alcoholism individuals. Pharmacologically relevant BACs are BACs that are in the range of 40 to 50 mg %, or higher. Chronic alcohol consumption resulting in these BAC levels (~ 0.7 g/kg/bout, or higher) results in the development of tolerance to alcohol-induced effects and signs of dependence/withdrawal upon cessation of alcohol access (169, 200). Recently, additional rat lines have been created with similar levels of alcohol consumption (91).

Gene expression in animal models of alcoholism: The study of molecular determinants of excessive alcohol consumption represents a major challenge. As outlined above, microarray studies have provided valuable new insight into gene regulation in genetically complex diseases such as alcoholism. Over the past decade, expression profiling has been extensively used to identify alcohol-responsive genes and pathways in animal models of alcoholism. A strategy commonly used among addiction researchers is to identify expression differences between strains of animals selectively bred for divergent drug-related phenotypes. However, limitations to this approach include the availability of resources to survey large numbers of genetically characterized strains and the lack of statistical power to identify small but reliable differences in gene expression within the brain. Access to large databases of expression data and meta-analytic approaches, already successfully used in the cancer field (201), have helped mitigate these obstacles (112, 113). The studies reported in the following subsections sought to identify constitutive differences in gene expression among diverse inbred strains or selectively bred alcohol-naïve animals, whereas others focused on the effects of alcohol intake, or administration, on gene expression compared with appropriate controls (Table 1).

Alcohol-naïve animal studies

Mice: An early expression profiling study examined inbred long-sleep (ILS) and short-sleep (ISS) mice, which show significant CNS-mediated differences in sleep time following a sedative dose of alcohol and have therefore been used as a rodent model for alcohol sensitivity. In this study, Xu et al. (118) found 41 genes that differed significantly between ILS and ISS mice. The identified genes could be functionally classified as oncogenes/tumor suppressors, ion channel/transport proteins, transcription factors and those involved in ubiquitination (118). However, in this study different array platforms, with a relatively small number of features per array, were utilized and the experimental design did not allow a formal statistical analysis and gene selection was based on arbitrary cut-off ratios or qualitative interpretation.

In a subsequent study, Kerns et al. (125) examined gene expression patterns across major components of the mesocorticolimbic dopamine pathway (NAc, PFC, VTA), a system known to be activated by alcohol and other addictive drugs (162), In their study, they compared expression profiles between control (alcohol-naïve) C57BL/6J (B6) and DBA/2J (D2) mice, known to differ markedly in a number of alcohol-related behaviors, as part of a larger experiment (125) (see “Alcohol-experienced animals” section below). In B6 and D2 control animals, more than 750 differences in gene expression were identified between strains, and the majority of these changes were observed in the PFC. Many of the genes differentially expressed between strains mapped to regions of mouse chromosomes 1 and 4, which are linked to QTLs for alcohol traits such as locomotor activation, acute withdrawal and preference (103, 202–205). These findings underscore the strength of gene expression studies when combined with known QTLs for complex traits. In a similar study, expression profiling was used to identify changes in the transcriptome between mice selectively bred for differential (high or low) acute functional tolerance (HAFT vs. LAFT) to alcohol’s effects (122). Multiple statistical procedures were used to ensure rigorous filtering criteria for the selection of differentially expressed genes. Similar to the Kerns et al. study (125),

identified genes had to be localized in QTLs associated with acute functional tolerance. The identified genes belonged to a signal transduction cascade involving the glutamate receptor delta-2 subunit, the Ephrin B3 ligand, and the NMDA receptor, as well as a transcriptional regulatory protein induced by activation of the NMDA receptor (zinc finger protein 179) and a protein that modulates downstream responses to NMDA receptor activation (peroxiredoxin). These authors postulated that these genes mediate acute functional tolerance through NMDA receptor phosphorylation and trafficking to the synaptic membrane (122).

Mulligan and colleagues (206) used a meta-analytic approach to identify candidate genes modulating alcohol consumption by combining several databases of expression data from genetic mouse models of voluntary alcohol consumption. In this study, 13 different strains of mice from five independent experiments originally performed in three different laboratories were combined. These studies utilized only alcohol-naïve animals and included selected lines bred for high and low drinking, inbred strains that differ in voluntary alcohol consumption, and an F1 hybrid strain between the C57BL/6J and FVB/NJ that shows the highest voluntary alcohol intake of any mouse genotype to date (195). Approximately 3,800 unique genes were identified that significantly and consistently differed between the mouse strains within each model of high or low alcohol consumption. The top 75 genes, ranked by effect size, fell into broad categories of cellular homeostasis and neuronal function. Several functional groups, including mitogen-activated protein kinase signaling and transcription regulation pathways, were found to be significantly overrepresented suggesting an important role in establishing a high level of voluntary alcohol drinking. In addition, the data from the general meta-analysis were further filtered by expression data from a mouse congenic line for chromosome 9, which contains genes associated with alcohol intake (207), to identify candidate genes within an alcohol-drinking QTL. Transcripts differentially expressed included beta-2-microglobulin; mannosidase, alpha, class 2B, member 1; sodium channel, voltage-gated, type IV, beta; microtubule-associated protein, RP/EB family, member 1; protein kinase C, epsilon; and somatostatin. Functionally, these genes are involved in immunity/cellular defense, glycosylation, ion-channel activity, microtubule, intracellular signaling and neuronal signaling, respectively. Overall, 20 putative novel quantitative trait genes underlying alcohol preference were identified.

Rats: Several studies examining differential gene expression between high and low alcohol-consuming rat lines have also been carried out (Table 1). Arlinde et al. (208) compared expression profiles between naïve AA and ANA rats. The cingulate cortex, NAc, amygdala (AMY) and hippocampus (HIP) of each line were analyzed revealing 48 differentially expressed genes between AA and ANA rats. Elevated hippocampal neuropeptide Y (NPY) was found in ANA rats in agreement with previous studies (209). A cluster of MAP-kinases indicating altered signal transduction was up-regulated within the NAc of the AA line, which is of particular functional interest. Within the AMY, a more loosely inter-related cluster of cytoskeleton-associated genes (including Gsk3b) suggesting differences in cytoskeletal properties and/or neuroadaptive function between the 2 lines. A study by Edenberg and colleagues (210) evaluated gene expression in the HIP of alcohol-naïve inbred alcohol-preferring (iP) and alcohol-non-preferring (iNP) rats (derived from the P and NP rat

lines, Indiana). The objective of this study was to test if there were innate differences in gene expression in the HIP, an area sensitive to the effects of ethanol, which may have a role in the development of tolerance to alcohol's effects (211). This study identified 129 differentially expressed genes which were functionally related to cell growth and adhesion, protein trafficking, regulation of gene expression, intracellular metabolism, intracellular signaling and synaptic function (210). Differences in the expression of these genes and/or systems may mediate differences in sensitivity to alcohol and/or in the development of tolerance to alcohol's effects between iP and iNP rats.

In a more comprehensive study by Worst et al. (212), gene expression was examined in the frontal cortex (FC) of rat strains genetically selected for alcohol self-administration preference, AA (Alko, alcohol-accepting) and P (Indiana, alcohol-preferring), or avoidance, ANA (Alko, alcohol-nonaccepting) and NP (Indiana, alcohol-nonpreferring), such that gene expression differences in the FC of AA and P vs. ANA and NP rats were examined. Among the detected genes, six demonstrated confirmable, differential expression following comparison of alcohol-naive AA and ANA rats. Specifically, the mRNA level for metabotropic glutamate receptor 3 (mGluR3) was down-regulated in the AA vs. ANA lines. In contrast, calcium channel subunit alpha2delta1 (cacna2d1), vesicle-associated membrane protein 2 (VAMP2), both syntaxin 1a and 1b (STX1a and STX1b), as well as syntaxin binding protein 1 (MUNC-18) mRNAs were found to be increased in the FC of AA vs. ANA rats. These genes are involved in neurotransmitter-release machinery and vesicle fusion. Thus, neurotransmission and/or synaptic machinery may differ between these rat lines. Of these genes, VAMP2 was the only one that was differentially expressed in the FC of P vs. NP rats, suggesting that the other gene differences found between AA and ANA rats may not be required for these alcohol-drinking phenotypes. Similar to the observation of brain region-specific heterogeneity in gene expression from post-mortem brain studies discussed above, there was no overlap in these genes compared with those identified in the HIP of inbred iP rats from the Edenberg study (210), and little overlap with genes identified in various brain regions of AA versus ANA rats from the Arlinde study (208).

A recent comprehensive gene expression study examined innate differences in multiple brain regions of iP vs. iNP rats (213). Gene expression differences were determined in the NAc, AMY, FC, caudate-putamen (CPU), and HIP of these strains. A significant number of gene expression differences were found in each of these brain regions. In general, the genes were functionally related to axon guidance, gliogenesis, regulation of programmed cell death, regulation of synaptic structure and function, as well as transmission of nerve impulses. However, the findings indicated that the greatest number of differences was not between the lines, but between the brain regions examined. Again, this indicates brain region-specific heterogeneity in gene, and possibly protein, expression differences, or changes, associated with alcohol self-administration or associated effects. The AMY showed the greatest number of differences in gene expression of the regions examined, although all five regions had a significant number of genes with significant differential expression. Taken together, the individual region and combined region analyses indicated that the expressions of genes involved in biologic networks of neurotransmitters, intracellular messengers, neuroplastins, neurotrophins, and transcription factors may all contribute to behavioral and neurobiological differences between the iP and iNP rats. Pathway analyses

revealed several differentially expressed genes involved in neuropeptide Y (NPY) neurotransmission. In addition, 13 of the 54 gene differences found in the AMY were located within established alcohol QTLs. However, since iP and iNP lines were inbred from P and NP, respectively, after many generations of selective breeding (91), the particular inbred strains used in this study may have subtle differences in characteristics from that seen in the parent lines of selectively bred P and NP rats or vice versa. Although evidence exists that similar differences found between the inbred strains iP/iNP are also present in the selectively bred P/NP lines, such as overt behavior, the development of rapid tolerance to alcohol's effects, along with the well-established alcohol-drinking phenotypes (200, 213).

Alcohol-experienced animals

Mice: Many researchers have used array profiling to identify line- and/or strain-specific changes in gene expression patterns after alcohol administration (Table 1). In mouse studies, gene expression levels were compared between B6 and D2 strains. However, researchers from the respective studies have examined different brain regions and used different alcohol administration methods. In two early studies, gene expression levels of the whole brain were compared following an acute (124, 214) or chronic (214) high doses of alcohol. However, interpretation of the findings from both of these studies is limited, since whole brain studies do not provide information about brain region-specific differences in alcohol-responsive expression patterns, and selection of significant gene differences was based on arbitrary cut-off ratios or qualitative analyses. This is especially relevant with the consistent observation of brain region-specific heterogeneity in the identified genes with expression differences.

In one of the earliest gene expression profiling studies, Daniels and Buck (119) investigated the effects of withdrawal after chronic and acute alcohol exposure on gene expression in the HIP. Although a limited number of genes were represented on the arrays used in this study, differentially expressed genes fell into several important signal transduction pathways. In D2 mice, withdrawal after acute and chronic alcohol treatment changed genes involved in, or associated with, mitogen-activated protein kinase, the Janus kinase/signal transducers and activators of transcription, as well as the Akt/phosphatidylinositol 3-kinase pathways. In contrast, the results indicated chronic withdrawal altered a different set of genes in the MAP kinase pathway of B6 mice. Together, these findings revealed that there are important differences in cellular adaptations to ethanol withdrawal between B6 and D2 strains.

As mentioned above, Kerns and colleagues (125) studied different regions of the mesocorticolimbic dopamine system (NAc, PFC, VTA) to assess strain differences in gene expression following an acute dose of alcohol. Alcohol regulated 307 genes in the PFC and NAc of B6 or D2 mice. In general, acute alcohol altered a larger number of genes in D2 compared to B6 mice. A striking finding was that more genes were up-regulated by alcohol than down-regulated in the PFC and NAc of D2 mice, whereas a greater number of genes were down-regulated than up-regulated by alcohol in these brain regions of B6 mice. In general, these authors indicated that the majority of alcohol-regulated genes involved neuroplasticity, although the regulation of discrete functional groups and pathways tended to be region-specific. For example, functional groupings for glucocorticoid signaling, neurogenesis, and myelination were found for the PFC; whereas neuropeptide signaling and

developmental genes including brain-derived neurotrophic factor (BDNF) were identified for the NAc; and retinoic acid signaling for the VTA. Once again, these findings illustrate the high degree of complexity and brain-region specificity of genes regulated by alcohol in animals with divergent alcohol-related phenotypes.

Gender-related factors can also play a crucial role in the regulation of gene expression by alcohol. In a study by Hashimoto and Wiren (215), mice with divergent withdrawal severity were used to characterize PFC gene expression differences associated with neuroadaptive response in both genders, following withdrawal from chronic alcohol exposure. Withdrawal Seizure-Prone (WSP) and -Resistant (WSR) mice, selectively bred from a genetically heterogeneous population, were chosen because of their suitability as animal model of neuronal hyperexcitability following alcohol exposure. Microarray analysis revealed a transcriptional response correlated with sex rather than with the selected withdrawal phenotype: In females, cell death and DNA/RNA binding related genes showed the higher changes, while in males protein degradation and calcium ion binding pathways were more affected by alcohol. The histopathological analysis of brain damage following alcohol withdrawal confirmed the microarray data, showing an elevated cell death in females but not male animals. The authors concluded that the disruption of the PFC inhibitory circuits may contribute to excessive drinking and self-sustaining nature of alcoholism (215). These findings are consistent with studies in human alcoholics showing that the susceptibility to brain damage associated with alcohol abuse is enhanced in females (216, 217).

Rats: Rat-based microarray studies examining direct strain-dependent differences in gene expression in response to alcohol are limited (Table 1). Rats develop a marked and long-lasting increase in voluntary ethanol intake after repeated cycles of intoxication and withdrawal, (218, 219). This drinking paradigm was used to identify alcohol-, and possibly withdrawal-, responsive genes in the cingulate cortex and AMY of Wistar rats (220). A small set of changed genes, mostly up-regulated, was reported in this model. The identified genes were associated with glutamatergic, endocannabinoid, and monoamine neurotransmission, all of which have been implicated in the development of alcohol dependence. Other identified pathways included the mitogen-activated protein kinase pathway. Another study focused on expression profiling from the dorsal HIP of inbred Lewis rats chronically exposed to alcohol (120). Alcohol-induced changes in gene expression were particularly prominent in three functional groups, including oxidative stress, dynein-associated polypeptides, dynamin-1 and membrane trafficking genes.

Three recent studies were conducted to determine the effects of binge-like alcohol drinking or oral operant alcohol self-administration and a subsequent withdrawal on gene expression changes in P rats. The first study (114) focused on the NAc, and the experimental design included three groups of alcohol-drinking P rats: a drinking-in-the-dark-multiple-scheduled-access (MSA) group, a continuous/daily access (CA) group, and an alcohol-naïve (W) group. The MSA and CA groups experienced 15 hours of withdrawal after the last drinking episode to keep time since ethanol exposure constant. CA resulted in 374 differentially expressed genes relative to the W group. Functional grouping involved negative regulation of protein kinase activity, anti-apoptosis, and regulation of G-protein coupled receptor signaling. Of these 374 genes, 43 were located within rat QTLs for alcohol consumption and

preference. Functional grouping included anti-apoptosis and increased transcription, suggesting a role for cellular protection in maintaining high alcohol intakes. Conversely, there was not a significant number (< than 5% of total number of genes represented on the array) of genes altered by MSA relative to the W group. These authors suggested that intermittent drinking, such as that induced by the MSA procedure, may result in tighter regulation of gene expression following repeated intermittent alcohol withdrawal periods. These findings (114) suggest that, under intermittent alcohol drinking conditions, gene expression levels may be maintained at a near normal steady-state, despite possible altered protein expression levels in the NAc. Similarly, the genes altered by CA drinking may be early acute withdrawal-responsive genes rather than purely alcohol-responsive genes.

In the second study (115), withdrawal time-course changes in gene expression were determined within two regions of the extended AMY [central nucleus of the AMY CeAMY and the shell of the NAc (shNAc)] after binge-like (MSA) alcohol drinking. The MSA groups included in this study experienced 1, 6, or 24 hours of alcohol withdrawal after the last drinking episode. There was not a significant number (< 5% of total number of genes represented on the array) of gene expression changes relative to W animals sacrificed at any individual time point. However, an overall effect across time-points was detected in both subregions. Functional grouping of the genes indicated there were several biological process categories common across the two regions (for example, synaptic transmission, neurite development), despite the fact that there were few transcripts in common across the two regions. Overall, these results indicate that binge-like alcohol drinking by P rats produces brain region-specific changes in the expression of genes involved in transcription, synaptic function, and neuronal plasticity. Therefore, binge-like alcohol-drinking may affect different aspects of common pathways across diverse brain regions (115).

In the third study (116), the effects of operant oral alcohol, saccharin or water self-administration on gene expression in the AMY and NAc of iP rats were examined. The vast majority of the detected changes occurred in the NAc vs. the AMY, 513 vs. 134 respectively. It is important to remember that the whole AMY and whole NAc were examined in this study. Thus, subregional differences may have masked some of the gene expression changes present especially within the AMY, a more heterogeneous structure than the NAc. Functional grouping of identified genes in the NAc of alcohol vs. water groups included ion transport, chemical homeostasis and synaptic transmission. It is noteworthy that when genes identified in the NAc from alcohol vs. saccharin animals were examined, functional grouping revealed 15 over-represented Gene Ontology categories. Some of these included ion/chemical homeostasis, endocytosis, myelination, neurogenesis and synaptic transmission. Genes identified in both the alcohol vs. water and alcohol vs. saccharin comparisons included caveolin 2, glutamic acid decarboxylase 1, GABA-A receptor beta 2 subunit, Homer 1 and neurexin 3 all of which are involved in synaptic transmission. This was the first study that identified candidate genes that are specific for alcohol reinforcement, such that differences were seen between the alcohol group and both the water and saccharin groups. The latter 2 groups were used to control for motoric and reinforcement behavior associated with an alternative palatable solution.

In a recent study, a convergent functional genomics approach was used to identify alcohol-responsive genes. This bioinformatics approach relies on the cross-matching of animal model brain gene expression data with human genetic linkage data, as well as human tissue data and biological roles data. Rodd and colleagues (221) successfully used this approach to analyze three animal model paradigms, based on inbred alcohol-preferring (iP) and alcohol-non-preferring (iNP) rats, and their response to treatments with alcohol. A comprehensive analysis of microarray gene expression data from five key brain regions (FC, AMY, CP, NAc and HIP) was carried out. For the alcohol self-administration components of the data, chronic free-choice consumption and operant intracranial self-administration into the VTA by iP rats were used. Overlapping expression data were then filtered using human genetic linkage data, human tissue data (post-mortem brain, lymphocytes and fibroblasts) and biological roles data. Analysis of the gene expression data identified about 3000 significantly changed genes across brain regions and experimental paradigms. The list of candidate genes was reduced by identifying those changed in all three experiments and those that were changed in at least two out of three experiments. An empirical probability scoring system was derived that combined expression data with the additional filters listed above to identify high-priority candidate genes. The highest-ranking genes (those changed in all three experiments) included CD81 molecule, nucleoporin like 1, phosphatidylethanolamine-binding protein and aldehyde dehydrogenase 6 family, member A1. This study demonstrated that large data sets of gene expression data from different species can be combined with behavioral and genetic data to identify genes or functional pathways that underlie alcohol-related phenotypes (221).

Tabakoff and colleagues conducted a similar study (109) using microarray data from serum for the clinical part of the experiment. This study used a genetical genomic approach that included phenotyping of HXB/BXH recombinant inbred (RI) rats combined with gene expression data filtered through alcohol-associated behavioral and gene expression QTL analyses. Importantly, this study identified the first QTL for alcohol consumption on rat chromosome 1. The human data were assessed for genetic polymorphisms using a custom genotyping array for 1,350 SNPs. Functional evaluation of the genes from the RI analysis revealed groupings associated with presynaptic GABA release, postsynaptic GABA receptor trafficking, and dopamine neuron activation. Functional evaluation of gene SNPs associated with alcohol consumption from the human data revealed groupings associated with GABA synthesis, GABA receptors and dopaminergic neurotransmission. It is striking that confirmatory findings between rodent and human data were obtained within the experimental design itself and not through meta-analytic approaches. This study exemplifies the use of multiple array platforms coupled with behavioral and genetic QTL analyses to obtain converging genetic findings putatively associated with alcohol abuse and dependence.

Gene expression profiling in cell cultures: Studies with the intact nervous system seem likely to be most relevant to understanding the mechanisms of alcohol and drug abuse-related behaviors. However, the use of expression profiling with in vitro neuronal culture models offers significant advantages for identifying details of cellular signaling actions and toxicity associated with drugs of abuse.

Thibault et al. (117) used microarrays to show that in SH-SY5Y neuroblastoma cells, ethanol treatment increased expression of 11 genes that were also increased by cAMP, one of which was dopamine-beta-hydroxylase (DBH), the enzyme required for the conversion of dopamine to norepinephrine. DBH is involved in noradrenalin synthesis and the microarray studies showed that ethanol induced several other genes involved in noradrenalin production. The importance of the in vitro coordinate regulation of multiple noradrenalin-related genes by ethanol is supported by in vivo studies implying that noradrenalin modulates ethanol consumption. Local infusions of norepinephrine into the hypothalamus increases ethanol consumption in rats (222), and DBH knockout mice show reduced ethanol preference (223). The results of this expression profiling study are also consistent with the in vivo and in vitro observations that ethanol alters cAMP signaling, function of the cAMP response element binding protein (CREB) and genes that are activated by the cAMP pathway (224–230). Finally, this report showed that ethanol regulated multiple genes related to oxidative stress or glutathione production. The relevance of this in vitro finding is underscored by numerous in vivo studies where ethanol generates a significant degree of oxidative stress in multiple organ systems (231–234). Such actions might be an important aspect of the mechanisms of alcohol-induced cellular toxicity in many organs (117) and subsequent effects in the central nervous system (163–167).

In another microarray pharmacogenomics study (121), in the same neuroblastoma cell line, it was also demonstrated that protein kinase A, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK), and casein kinase II inhibitors blocked the increase in dopamine-beta-hydroxylase expression as well as a large subset of additional ethanol-responsive genes. These studies indicate that important information on mechanisms of cellular/organ toxicity can be obtained from the integration of expression profiling studies with in vitro models of excessive alcohol exposure, offering the potential for novel mechanistic rigor and physiological relevance.

MicroRNA regulation of gene expression—The majority of the RNA in a cell does not code for proteins, but is non-coding regulatory RNA that orchestrates the function of the cell. MicroRNAs (miRNAs) are small, non-coding oligonucleotides with an important role in regulation of gene expression at the level of translation, and they can also regulate mRNA expression. Non-coding miRNAs are emerging as “master regulators” of gene expression and may underlie many of the widespread genomic changes produced by chronic alcohol consumption. Recent studies have revealed that miRNAs play critical roles in regulating diverse biological processes such as neuronal differentiation, developmental timing, synapse function, and neurogenesis (235–238). With regard to alcoholism, miR-9 was found to promote splice variations in the mRNA coding for the pore-forming alpha-subunit of the BK channel (239), a large-conductance calcium- and voltage-activated potassium channel. Alcohol exposure causes an increase in miR-9 expression which then results in a rapid degradation of one splice variant of the alpha-subunit, causing reorganization of transcripts to form an alcohol-resistant BK channel (239). This is just one example of regulation of a miRNA by alcohol exposure.

A single miRNA can target hundreds of mRNA transcripts for either translation repression or degradation, and conversely, individual mRNA transcripts may be regulated by the co-

ordinate action of multiple miRNAs (240). However, there is little direct experimental evidence regarding the normal function of many human miRNAs or their role in disease. There is considerable regulatory potential of miRNAs since there are over 700 in humans (http://www.mirbase.org) (241) that often act as master regulators, capable of silencing the expression of large collections of target genes. Despite their biological importance, the determination of miRNA targets is a major challenge. These target genes are defined by short sequences in their 3′ untranslated regions (UTR) that are complementary to a given miRNA, and although bioinformatic tools have improved the unbiased prediction of miRNA binding sites, different algorithms produce divergent results with high false-positive rates. This problem was recently overcome by the combination of HITS-CLIP (high throughput sequencing of RNAs isolated by crosslinking immunoprecipitation) with bioinformatics to produce a map of functionally relevant miRNA binding sites (242).

Growing evidence suggests that alcohol exposure can change miRNA expression profiles (243); given that miRNAs regulate many cellular functions, it is reasonable to expect that they play a significant role in mediating the effects of alcohol, such that alcohol alters miRNA levels and miRNA-regulated systems that may determine effects such as ethanol-induced tolerance, gut leakiness, and neural stem cell proliferation and differentiation (239, 244, 245). In a recent study using post mortem human brain, a number of miRNAs were significantly up-regulated in alcoholics compared to controls, and, interestingly, the miRNAs seemed to work in a combinatorial manner to alter gene expression patterns. Correlation between the predicted targets of these miRNAs with actual mRNA expression profiles supports the hypothesis that alcohol alters gene expression patterns via miRNA-mediated mechanisms and that miRNAs act co-ordinately to alter the expression of these target mRNAs (Mayfield et al., unpublished data). Much of this work has utilized a ‘top down’ approach where an alcohol phenotype is explored and the underlying molecular basis is associated with changes in the abundance of miRNAs produced by alcohol exposure. The current tools for miRNA expression profiling are limited by the requirement for a priori knowledge of miRNA sequences and evidence suggests that novel miRNAs involved in mediating the action of alcohol in the brain remain undiscovered. Additional studies with complementary techniques will help to confirm the changes in miRNA expression induced by alcohol exposure, as was done for cocaine-induced adaptive changes in a recent study (246). With the availability of high-throughput next generation sequencing, the technical drawbacks of probe-based methodologies can be overcome. Direct miRNA sequencing provides information about SNPs as well as post-transcriptional RNA editing, single nucleotide additions, and variation in miRNA length (247–249).

Use of cutting edge technologies: Next gen sequencing—The alcohol addiction research has been reshaped by a number of increasingly sophisticated new genetic tools (selected lines, recombinant inbred strains, QTL analysis, gene expression arrays, SNP maps and so on). Nevertheless, we may not be able to define the genetics of dependence until we have a better comprehension of how genes can interact with environmental variables to influence drug responses and the related behaviors (6).

Although DNA microarrays have advanced our understanding of complex cellular function, the reliance of microarrays on hybridization kinetics results in several technical limitations.

For example, knowledge of the sequences being probed is required, distinguishing similar sequences is difficult because of cross-hybridization, and the relatively narrow dynamic range of the signal limits sensitivity. New technologies, termed next generation sequencing, are free of the limitations inherent to microarrays. Next generation sequencing is unique since it allows the detection of all known and novel RNAs present in biological samples without bias toward known transcripts. In addition, the expression of all coding and non-coding RNAs (including microRNAs), alternative splicing events, and expressed single nucleotide polymorphisms (SNPs) can all be identified in a single experiment. This significant shift in throughput and pricing makes low-cost access to whole genomes possible, but more importantly, it expands sequencing applications far beyond traditional uses (250). Such non-traditional uses include sequencing the transcriptome (RNA-Seq), the population of RNA molecules in a cell or living tissues, providing detail on gene structure, alternative splicing events, expressed SNPs (251–253), and transcript size, while also quantifying the absolute abundance of genes, with greater sensitivity and dynamic range than the competing cDNA microarray technology (254). The detailed whole genome information which is only available from this approach can be combined with clinical and/or other phenotypic data to provide increased understanding of basic biological processes and a more integrated view of cellular function and regulatory networks.

Protein studies of clinical relevance

Protein expression studies: The development of alcohol dependence, tolerance, and addiction is linked with various neuroadaptations and changes in protein expression levels. For example, repeated alcohol administration induced an up-regulation in the iGluR and mGluR expression and function, plus affected glutamate receptor trafficking to, and clustering within, the plasma membrane (255, 256). Recent immunoblotting studies have examined how repeated alcohol exposure could affect the protein expression of Homer isoforms in the NAc (256). In these studies, alcohol-experienced C57BL/6J mice showed a marked and selective up-regulation in Homer2 protein expression within the NAc, following various alcohol treatment regimens (255). In addition, alcohol up-regulated the NAc expression of the members of the mGluR-Homer-NMDA signaling complex and increased the activation of mGluR-Homer-mediated signaling cascades. The authors propose that taken together, their immunoblotting and behavioral genetic studies implicate an up-regulation in NAc Homer2 expression as a crucial cellular adaptation to alcohol, which facilitates alcohol-induced changes in behavior and alcohol drinking (256). A similar study examined protein levels for group 1 mGluRs, NR2 subunits and Homer proteins in the NAc core (coNac) and shNac, as well as the basolateral AMY (blAMY) and ceAMY of P rats experiencing intermittent (IA) or continous access (CA) to alcohol with brains extracted either 24 hrs or 28 days after their last drinking episode (257). The primary changes, relative to water control-values, were observed in the coNAc and ceAMY, consistent with region-specific changes found in other studies. In addition, most of the changes observed in the ceAMY, but not coNAc, were observed at 24 hrs and 28 days post-alcohol drinking, indicating long-term changes were region-specific as well. Thus, the role of NR2 subunits and Homer proteins in mediating alcohol-drinking behavior is region-specific and present across species.

More recently, the same group showed additional evidences for Homer2 and mGluR5 implications in regulating alcohol reward in an elegant study (258). By using the scheduled high alcohol consumption (SHAC) model of binge alcohol drinking, the researchers demonstrated that the augmented mGluR5-Homer2-PI3K signaling in the NAc predisposes a high binge alcohol-drinking phenotype. The binge drinking paradigm elevated the Homer2a/b protein expression and increased PI3K activity in the NAc. These data were validated by additional experiments, by showing that the site-directed pharmacological and transgenic interruption of the mGluR5-Homer2-PI3K signaling pathway reduced the extent of drinking in the SHAC mice model of binge alcoholism (258). These data highlight the importance of this pathway in regulating the binge-like alcohol consumption in mice.

Shotgun proteomics studies: The basic concept of shotgun proteomic analyses is the identification of proteins in complex mixtures derived from tissues or cells by combining high performance liquid chromatography (HPLC) with mass spectrometry (MS). Since an extract can easily contain several thousand proteins at a wide range of concentrations, this approach has become possible only through the development of automated processes. Proteomic studies have been widely used in different fields, and researchers increasingly have begun to use proteomic analyses to investigate alcohol's effects on the brain over the past few years. Such studies can potentially enhance our understanding of the effects of alcohol abuse in a meaningful way. Compared with the genomic studies, proteomic investigations are much more challenging for several reasons. Indeed, the proteome is much larger than the genome: A large diversity in proteins results from differential gene splicing and posttranslational modifications (PTMs). Plus, protein expression levels are vastly more variable than gene expression levels (65), the same amount of protein is not translated from each gene, and some proteins can be expressed in distinct cell types only. Furthermore, it is common for a variety of proteins to be present only in incredibly small amounts, especially those with regulatory functions. Thus, their detection is challenging, relative to background noise from all of the other proteins expressed in much greater amounts at the same time in the same cells or tissues.

Proteomics in human post-mortem brain: Several studies have been conducted using autopsy samples (Table 2). Lewohl and colleagues (259) studied the superior frontal cortex (SFC) proteome of long-term alcoholics and healthy control subjects. Proteins from the healthy and alcoholic subjects were compared for differential expression by using 2-DE. In addition, the investigators were able to identify 63 of the differentially expressed proteins, with the use of matrix-assisted laser desorption ionization (MALDI) tandem mass spectrometry (MS/MS). The researchers showed how proteomic studies can be conducted on autopsy samples to identify candidate proteins that are affected by long-term alcohol use and whose exact roles can be analyzed further (259).

More recently, in the same laboratory (260), synaptosomal preparations from post-mortem human brain of chronic alcoholics and non-alcoholic controls were compared using 2-DE, and superior frontal gyrus (SFG) as well as occipital cortex (OC) were analyzed from both groups. Among the observed differentially regulated proteins, a selection was identified by MALDI-time of flight (ToF) MS revealing proteins involved in vesicle transport,

metabolism, folding and trafficking, and signal transduction: All of the identified proteins can potentially influence synaptic activity. This study confirmed a number of proteins previously related to alcoholism as well as uncovered novel alcoholism-affected proteins. Alcoholism produced alterations in proteins involved in synaptic transmission, and the authors hypothesized that the reduction of dynamin-1 in the alcoholics SFG may contribute to the alcoholism's neurodegenerative effects and to its general disruption of cognitive function (260).

Several alcohol-sensitive brain regions from uncomplicated and hepatic cirrhosis-complicated human alcoholics have been analyzed in many studies by the Matsumoto group (261–266). Their studies represent a good example of how high-throughput neuroproteomics approaches can potentially dissect the mechanisms of complex brain disorders, and how every brain region responds in a significantly different manner to chronic alcohol abuse. Autopsy samples obtained from alcoholics with and without cirrhosis, one abstinent alcoholic, and nonalcoholics were used to compare the proteomes of the prefrontal white matter (PWM) (261), an area particularly susceptible to alcohol-induced brain damage and shrinkage. Among the 60 differentially expressed proteins detected, 18 proteins were identified, which included some enzymes involved in energy production in the cell, as well as some proteins that have been previously associated with alcohol-related disorders and brain damage (261). In other studies, apparent abnormalities in thiamine-related biochemical pathways were observed in several brain regions, such as the dorsolateral prefrontal cortex, the frontal part of the corpus callosum, and the cerebellar vermis in uncomplicated alcoholics, suggesting that the reduction of the vitamin B1 might be associated with brain damage, even without the signs of Wernicke-Korsakoff Syndrome (WKS). Plus, in the frontal and posterior subregions of the corpus callosum and in the cerebellar vermis, significant differences in protein expression profiles between uncomplicated and complicated alcoholics with hepatic cirrhosis were identified. Finally, significant changes in the level of glutamine synthetase expression were observed in the HIP. The authors suggest that hepatic factors such as ammonia have significant additive influences on brain protein expression, which might lead to the structural changes and/or damage in these brain regions (266).

Although these studies suggest that chronic alcohol consumption can directly alter the levels of several important brain proteins, it should be pointed out that such changes may also result from other concomitant conditions. For example, changes in brain gene expression are greater in alcoholics with cirrhosis than in those without cirrhosis (163), suggesting that such changes might contribute to the more severe brain dysfunction in individuals with liver disease.

Proteomics in animal phenotypes: Some researchers have applied the shotgun proteomics approach to some animal models of alcohol consumption (Table 2). By using 2-DE and MALDI-ToF MS, the proteomes of relevant brain regions from P and NP rats have been compared (267). 70 proteins were identified whose expression differed significantly between the two rat lines, and the largest differences were found for various proteins involved in signaling pathways. Furthermore, protein expression was generally lower in the P rats than

in the NP rats. Again, chronic alcohol use appeared to reduce the expression of the majority of proteins studied, a common theme in the majority of the proteomic studies.

A similar study (268) investigated the effect of chronic alcohol drinking on protein expression levels in the NAc and AMY of iP rats experiencing either continuous access (CA) or binge-like (DID-MSA) access to alcohol. The results indicated that DID-MSA affected protein levels in the NAc to a greater extent than CA, whereas CA appeared to have a greater effect on the AMY than DID-MSA, again indicating region-specific changes induced by alcohol and/or type of alcohol access. In general, the proteins could be grouped into functional categories, including chaperones, cytoskeleton, intracellular communication, membrane transport, metabolism, energy production, and neurotransmission. The authors concluded that the diverse pattern of protein expression changes (only 2 proteins were changed in both regions, annexin V and tropomyosin, gamma) between the NAc and AMY might reflect differences in neuroanatomical and/or functional characteristics associated with ethanol self-administration and possibly withdrawal, between the two brain structures.

In a recent study (269), the effects of repeated systemic administration of a moderate dose of ethanol was determined on protein expression in the shNAc of alcohol-preferring (P), alcohol-non-preferring (NP) and Wistar (W) rats. Rats were injected for 5 consecutive days with either saline or ethanol and experienced 24 hours of withdrawal after the last injection. A liquid chromatography-MS procedure was used to assess the ethanol- induced changes in the proteome. Ethanol altered the expression levels of a higher number (about 5 times) of proteins in NP rats, compared to P and W rats. Few of the changes observed with ethanol treatment for NP rats were observed for P and W rats. Many of the changes occurred in calcium-calmodulin signaling systems, G-protein signaling systems, synaptic structure and histones. Approximately half of the changes observed in the shNAc of P rats were also observed for W rats. Overall, this study showed a unique response to ethanol within the shNAc of NP rats compared to P and W rats; this unique response may reflect changes in neuronal function in this brain area that could contribute to the low alcohol drinking behavior and/or higher sensitivity to alcohol exhibited by NP rats (269).

Interaction proteomics approaches: Protein-protein interactions are of central importance for virtually every process in a living cell. Proteins operate in harmony with other proteins by establishing complexes and networks to orchestrate the multiplicity of processes that impact cellular function. For example, protein interactions participate in many physiological processes, and are crucial for neurotransmission in the brain. The release of neurotransmitter molecules responsible for signaling among neurons involves regulated protein-protein interactions (270). Ion channel or neurotransmitter transporter proteins located in the synaptic membrane are regulated by complex protein interactions (113, 271–276). Therefore, identification and characterization of these protein-protein interactions can potentially improve our understanding of the processes that occur during normal neurotransmission and might provide new insights into cell function adaptations in the presence of alcohol. Updated information about these interactions will certainly increase our understanding of diseases and, importantly, could provide the basis for new therapeutic approaches. Indeed, newly identified protein-protein interactions may represent novel targets

for drug development. This approach has emerged as an important area in medication development (277, 278).

There is growing evidence that functionally-related gene expression patterns often predict protein-protein interactions (279–281). Gene expression studies suggest that alcohol alters the expression pattern of a number of genes required for normal synaptic function. Proteins encoded by these genes are important for a variety of synaptic events, including neurotransmitter vesicle transport and targeting, motor proteins involved in trafficking and targeting of synaptic proteins, and scaffolding proteins. Thus, excessive alcohol consumption likely alters protein complexes required for normal synaptic transmission, and protein interactions may therefore represent important sites in the search for new medications to treat complex diseases such as alcoholism.

There is limited knowledge about alcohol's direct action on synaptic proteins in the context of multiple interacting partners; while there is interest in identifying the accessory proteins that interact with synaptic proteins, relatively few have been identified and confirmed.

In a recent study (282), interaction proteomics was used to examine synaptic protein complexes isolated from cortical membranes prepared from alcohol-naïve C57BL/6J mice. To test protein-protein interactions, immunoprecipitation experiments were performed by using as baits some synaptic proteins encoded by genes whose expression is regulated by excessive alcohol consumption: Syntaxin-1A (212), synaptosome-associated protein 25 (163), vesicle-associated membrane protein 2 (212), dynamin-1 (120), and the BK channel (239). Subsequent Western blots and mass spectrometric analyses confirmed known, and identified novel, interacting protein partners in the co-immunoprecipitates. Remarkably, the BK channel complex involved many alcohol-sensitive proteins, including dynamin-1, syntaxin-1A, syntaxin binding protein 1, and members of the kinesin superfamily (282). Thus, given that the BK channel is a well-established alcohol target, important in behavioral and molecular tolerance (239), and many of its interacting partners are translated from genes perturbated by alcohol as well, future studies will certainly focus on its protein complex.

Some other important studies have been conducted for different neurotransmitter systems that are known to be affected by alcohol. For example, Husi and colleagues (283) characterized the protein complex making up the N-methyl-D-aspartate (NMDA) receptor by using interaction proteomics. This study led to the identification of 77 different proteins as interacting partners in the NMDA receptor complex, with a various range of functions such as binding glutamate and initiating intracellular signaling processes (283).

Dopaminergic neurons are another target of alcohol action in the brain, and the activity of the dopamine transporter (DAT) is regulated by multiple signaling mechanisms, at least some of which are likely to involve protein-protein interactions. An interaction proteomics approach was used by Maiya and colleagues (113) in an attempt to identify the DAT interacting protein partners. Using immunoprecipitation followed by 1D gel electrophoresis for the separation of the co-precipitate, individual partner proteins isolated from the gel were identified by MS analysis. The dopamine transporter was thus found to be associated with 20 proteins with diverse cellular functions that could be classified as signaling proteins,

trafficking proteins, cell adhesion molecules, ion channels, cytoskeletal proteins, metabolic enzymes, and extracellular matrix-associated proteins. Particularly, DAT was found to specifically interact with the voltage gated potassium channel Kv2.1, and the synaptic proteins synapsin 1 and dynamin 1, involved in regulating neurotransmitter release and recycling. An in silico analysis was also performed in order to evaluate the biological significance of these interacting proteins as a group. The correlation between the expression levels of the genes encoding the various interacting proteins was greater than would be predicted by chance alone, suggesting common regulatory mechanisms (113).

In summary, select clusters of genes predict meaningful networks of interacting proteins that are sensitive to the effects of alcohol and may represent potential sites important for medication development. The elucidation of novel mechanisms by which alcohol regulates complexes of interacting proteins will represent a significant contribution to the field.

IV. Translational strategies and need for continued biomarkers development

Translational research involves applying discoveries generated during research in the laboratory to the development of trials and studies in humans. The importance of translational research and medication development is obvious from the fact that there are only three FDA-approved drugs (disulfiram, naltrexone, acamprosate) for treatment of alcohol dependence and none of these have shown strong, consistent, effects in clinical trials and are not widely used in the treatment of alcoholism. A review of this complex and controversial area is beyond the scope of this introduction, but are discussed with the data from a large multi-site study showing limited efficacy for naltrexone and no effect of acamprosate on cessation of alcohol abuse (47). Given the need for more effective medications, the question is how we might use emerging research on the neurobiology of alcohol to rationally identify new candidates for medication development. As outlined in previous sections, the key steps are target identification and validation, followed by translation to selection of target-specific ligands, and testing of these ligands in both physiological and behavioral models.

Current biomarkers

Biomarkers with diagnostic and prognostic value play a pivotal role to the addiction field. Indeed, the successful treatment of most diseases relies heavily upon early detection. Most individuals with alcohol or drug dependence or use problems usually evade detection until severe medical, legal, or social problems happen (284). The discovery of reliable blood-based molecular markers of alcohol dependence and use would mark a milestone for alcohol research and offer a great benefit for predicting the disease even without knowing the role of the markers in the disease process. Once biomarkers are discovered, their functional significance in alcoholism can be studied, and this may lead to new promising drug treatments for the disease.

The most direct way to determine alcohol consumption is to measure the presence of alcohol or its metabolites in body fluids or breath. Such measures can be useful under certain

conditions, and therefore research has successfully worked on the development of diagnostics of acute alcohol consumption for decades. Several accurate methods can determine alcohol concentration through breath, urine and blood. Small inexpensive instruments are commonly used by law enforcement, medical, and security personnel. However, the development of reliable diagnostic tools that can retrospectively examine alcohol intake across days or weeks remains more challenging, since the relatively short half-life of alcohol in the blood (284, 285). Nevertheless, researchers have identified biomarkers of alcohol intake with longer ranges of assessment than direct alcohol measures of breath and body fluids. These biomarkers measure alcohol consumption indirectly, by detecting tissue damage or other physiological reactions to heavy drinking over time (73, 286).

The most common traditional marker is gamma-glutamyltransferase (GGT) (287). The liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are also measured as biomarkers of heavy alcohol consumption in routine screening for liver damage (288). An increase in the mean corpuscular volume (MCV), which is the size of red blood cells, represents another indication of chronic heavy drinking (289).

Currently, the most specific serum marker of chronic, heavy alcohol use is carbohydrate-deficient transferrin (CDT) (288, 290–293), but its detection test has a low sensitivity in the general population, and thus it is not a reliable candidate for predicting either heavy alcohol use or for diagnosing alcohol abuse or dependence (294). Furthermore, traditional blood biomarker tests for predicting alcohol use have not been universally accepted or generally adopted in clinical practice because their accuracy, sensitivity, and specificity are frequently lower than required for diagnostic purposes (73, 286), since they depend on many factors including age, gender and the population being studied (284).

Recently developed biomarkers include serotonin metabolites 5-hydroxytryptophol (5-HTOL) and glucuronidated 5-hydroxytryptophol (GTOL), elevated during alcohol consumption (295, 296). Direct measurement of alcohol metabolites has been also proposed as biomarker: For example, ethyl glucuronide (EtG) has a longer detection time in the urine than in the blood (14 to 24 hours) (297–299). The methodology for the measurement of this metabolite in the hair is currently under development, but EtG can already be detected in the hair up to several months after drinking has stopped (286, 300). The possible implications for such a biomarker include forensic purposes, the monitoring of abstinence among individuals convicted of driving while intoxicated (301) and women who are at risk for drinking during pregnancy (302, 303). Other promising alcohol metabolites are phosphatidylethanol (PEth), ethyl sulfate (EtS), fatty acid ethyl esters (FAEEs), and acetaldehyde adducts (286).

Finally, advances in technology are leading to the development of new sophisticated noninvasive alcohol biosensors that are able to quantitatively measure the amount of drinking and also to determine when drinking has occurred. For example, Giner WrisTAS (285) and SCRAM (Secure Continuous Remote Alcohol Monitor) (304) are bracelets worn around the wrist or the ankle, respectively, which electrochemically measure transdermal alcohol vapor and continuously record the drinking schedule.

One of the future goals of alcohol research would be to develop a reliable blood test for diagnosing alcoholism in the general population. This would achieve another milestone not only for the treatment of individuals but for the benefit of society. A reliable diagnostic assay for alcohol dependence could augment treatment and prevention programs. A test for recent heavy drinking could be part of future drug screenings before and during employment and could be invaluable for screening employees such as airline pilots, taxi, truck, and school bus drivers. The test could also be used for obtaining or regaining driver's licenses. Importantly, the commercialization of a blood assay would lead to faster and wider spread detection of alcoholism. Once detected, treatment to disrupt the progression of the disease can be implemented.

Biomarkers development strategies

New high throughput technologies such as proteomics, genomics, and metabolomics will increase the possibility of discovering biomarker panels or signatures with the potential to be more sensitive and specific. Preclinical researchers are searching for new alcohol consumption biomarker signatures to monitor either alcohol intake or alcohol-induced organ damage, and clinicians are beginning to appreciate how these markers might provide hints to the alcohol pathophysiology on organs such as liver, heart, and lungs. In addition, better appreciation for the usefulness of an unbiased identification of fluctuating amounts of alcohol consumption has grown in the clinical, employment, and forensic areas (305).

Proteomic approaches are being used to search for biomarkers of alcoholism, which could be represented by proteins that differ in abundance between alcoholics and nonalcoholics and which can be easily measured to assess whether a person has been drinking alcohol recently or is an alcoholic. Kasinathan and colleagues (306) have used such approach to search for urinary biomarkers of alcohol intake, which could be useful for monitoring alcoholics during treatment and for identifying people who are at risk for alcoholism. Using 2-DE, the proteomes of the urine of alcohol-treated and control rats were compared, and several proteins were present in the urine of alcohol-treated animals but not in the urine of control animals. With the subsequent tandem MS analyses, one of these proteins was identified as an enzyme called transferrin 2. This modified form of transferrin, called carbohydrate-deficient transferrin, can be measured using blood tests, and it is already being used by researchers and clinicians as a biomarker for chronic alcohol consumption (307). Further analyses are needed in order to determine whether these findings can also apply to humans and whether the modified transferrin can be reliably used as a biomarker for alcoholism.

In another study, Freeman and colleagues (308) analyzed serum proteins from cynomolgus monkeys subjected to a self-administration paradigm of alcohol, trying to identify potential biomarkers for alcoholism by looking at the differences between the control and the alcoholic monkeys. Using an MS technique called surface-enhanced laser desorption ionization/ time of flight (SELDI-ToF), the researchers detected two proteins that were differentially expressed in the two groups. These two proteins were identified as apolipoprotein AI, up-regulated in alcoholic animals, and apolipoprotein AII, up-regulated only in some animals. Both proteins are components of high-density lipoprotein (HDL),

commonly known as “good” cholesterol. Importantly, this study demonstrated that non-human primates can serve as a reliable model for the identification of biomarkers in alcohol research, given that it showed consistency of results with previously published data on human subjects (267). Studies involving non-human primates might be promising since they do not present some of the limitations associated with the human subjects, such as inconsistent self-reporting of alcohol intake, variations in diet, and other individual differences between subjects.

Perspectives for medication development

In the past, alcohol dependence was perceived as a moral failing and predominantly treated with non-medical therapies meant to punish the behavior; however, thoughts and attitudes about AUDs have changed considerably (309). Although there is still some hesitance in considering AUDs strictly as a medical problem, in the future alcohol dependence will likely be seen as a brain disease similar to other psychiatric disorders. With increased attention on its genetic predisposition and the potential long-lasting and/or permanent changes in neurotransmission systems, AUD treatment will be eventually integrated into the medical system. Indeed, since AUD etiology includes genetic, neurobiological, psychological, and environmental factors (310), the ideal therapeutic approach has to combine both pharmacological and psychosocial treatments (49). Nevertheless, the diagnosis of AUDs can be biased and problematic and the current treatments are mostly psychosocial therapies, which are conducted outside medical settings and lack of universal effectiveness (309). Treatment trials are increasingly adopting biomarkers to help in the evaluation of standard interventions and new medications (305). In alcohol research, biomarkers have the potential to serve as trait markers of AUD phenotypes, and the identification and categorization of diverse alcohol dependence phenotypes will provide models for the multiple subtypes of alcohol dependence (284). This will allow clinicians to identify patients who are likely to respond positively or negatively to specific treatments and medications. Thus, biomarkers are expected to have a central role in personalized medicine for AUDs (286).

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Table 1

Summary of the gene expression studies described in section III (see text for details).

Model	*Exposure*	*Strain/line*	*Source*	*Gene categories, pathways, genes*	*Ref.*
human	alcoholic		PFC	myelination, protein trafficking, ubiquitination, mitochondrial function	(143, 144, 147)
human	alcoholic		PFC	myelination, ubiquitination, apoptosis, cell adhesion, neurogenesis, neural disease, presenilin 1, transferrin, GABA-B receptor 1	(83)
human	alcoholic		PFC	DNA-binding proteins (transcription factors and repair proteins), mitochondrial proteins, neuroprotection, apoptosis	(148)
			NAc	synaptic vesicle formation, cytoarchitecture	
human	cirrhotic		PFC	astrocytes-related	(163)
mouse	naïve	HAFT/LAFT	whole brain	NMDA receptor phosphorylation, synaptic trafficking, glutamate receptor delta-2 subunit signal transduction cascade, ephrin B3 ligand, NMDA receptor	(122)
mouse	naïve	multiple	whole brain	mitogen-activated protein kinase signaling, transcription regulation pathways, beta-2-microglobulin, mannosidase, alpha2B 1, Scn4b, MAP RP/EB 1, protein kinase C epsilon, somatostatin, immunity/cellular defense, glycosylation, ion-channel activity, microtubule, intracellular signaling, neuronal signaling	(206)
rat	naïve	AA/ANA	HIP	neuropeptide Y (NPY)	(208)
			NAc	MAP-kinases	
			AMY	cytoskeleton, Gsk3b	
rat	naïve	iP/iNP	HIP	cell growth/ adhesion, protein trafficking, regulation of gene expression, intracellular metabolism, intracellular signaling, synaptic function	(210)
rat	naïve	AA/ANA, P/NP	FC	mGluR3, calcium channel subunit alpha2delta1 (cacna2d1), VAMP2, syntaxin 1a / 1b, syntaxin binding protein 1 (MUNC-18)	(212)
rat	naïve	iP/iNP	various	axon guidance, gliogenesis, regulation of programmed cell death, regulation of synaptic structure and function, transmission of nerve impulses, biologic networks of neurotransmitters, intracellular messengers, neuroplastins, neurotrophins,transcription factors	(213)
mouse	alcohol inhalation	B6/D2	HIP	mitogen-activated protein kinase, Janus kinase/signal transducers / activators of transcription, Akt/phosphatidylinositol 3-kinase pathway, MAP kinase pathway	(119)
mouse	acute alcohol injection	B6/D2	PFC	glucocorticoid signaling, neurogenesis, myelination	(125)
			NAc	neuropeptide signaling, developmental genes, BDNF	
			VTA	retinoic acid signaling	

Model	*Exposure*	*Strain/line*	*Source*	*Gene categories, pathways, genes*	*Ref.*
mouse	alcohol inhalation	WSP/WSR	PFC	cell death, DNA/RNA binding (females); protein degradation, calcium ion binding (males)	(215)
rat	intermittent alcohol	Wistar	AMY	glutamatergic, endocannabinoid, monoamine neurotransmission, mitogen-activated protein kinase	(220)
rat	chronic alcohol	Lewis	HIP	oxidative stress, dynein-associated polypeptides, dynamin-1, membrane trafficking genes	(120)
rat	MSA/CA/W	P	NAc	protein kinase activity, anti-apoptosis, regulation of G-protein coupled receptor signaling	(114)
rat	MSA/W	P	AMY, NAc	synaptic transmission, neurite development	(115)
rat	self adm. alcohol/ water	iP	NAc	ion transport, chemical homeostasis, synaptic transmission	(116)
	self adm. alcohol/ saccharine	iP	NAc	ion/chemical homeostasis, endocytosis, myelination, neurogenesis, synaptic transmission	
	self adm. alcohol/ water, alcohol/ saccharine	iP	NAc	caveolin 2, glutamic acid decarboxylase 1, GABA-A receptor beta 2, Homer 1, neurexin 3, synaptic transmission	
rat	self-adm. alcohol	iP/iNP	various	CD81 molecule, nucleoporin like 1, phosphatidylethanolamine-binding protein, aldehyde dehydrogenase 6 family	(221)
rat	2 bottle choice alcohol	HXB/ BXH	serum	presynaptic GABA release, postsynaptic GABA receptor trafficking, dopamine neuron activation, GABA synthesis, GABA receptors, dopaminergic neurotransmission	(109)
cultured cells	chronic alcohol	SH-SY5Y	neuroblastoma	noradrenalin production, dopamine-beta-hydroxylase (DBH), cAMP signaling, CREB function, cAMP pathway	(117)

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Table 2

Summary of the expression proteomics studies described in section III (see text for details).

Model	*Exposure*	*Strain/ line*	*Source*	*Protein categories, pathways, proteins*	*Ref.*
human	alcoholic		SFC	α- and β-synuclein, α- and γ enolase, β-neoendorphin-dynorphin, tubulin, pyruvate dehydrogenase, peroxiredoxin 2, heat-shock cognate 71 kDa, tropomyosin 2, tyrosine 3-monooxygenase	(259)
human	alcoholic		SFG, OC	synaptic transmission, vesicle transport, dynamin 1, metabolism, folding, trafficking, signal transduction	(260)
human	cirrhotic		PWM	energy production	(261)
human	cirrhotic		various	thiamine-related biochemical pathways	(262–265)
human	cirrhotic		HIP	glutamine synthetase	(266)
rat	naïve	P/NP	HIP, NAc	signaling pathways, cellular retinoic acid-binding 1, calmodulin-dependent kinase	(267)
rat	DID-MSA/CA	iP	NAc, AMY	chaperones, cytoskeleton, intracellular communication, membrane transport, metabolism, energy production, neurotransmission	(268)
rat	alcohol injections	P/NP/Wistar	NAc	calcium-calmodulin signaling systems, G-protein signaling systems, synaptic structure, histones	(269)